



ATTACHMENT 1

US Patent Application 10/664,970

Further examples for the generation and isolation of monoclonal antibodies being specific against VASP phosphorylated at position or phosphorylated at position 239

Example 1

Isolation of a monoclonal antibody against phosphorylated VASP at position 157

A phosphorylated peptide and a non-phosphorylated peptide, each of which encompasses the peptide sequence ERRS157 NAG around serine 157, are synthesized using an Applied Biosystems peptide synthesizer (Model 431A) in accordance with the Fmoc chemistry which is familiar to the skilled person. The phosphoserine in the phosphorylated peptide is incorporated during peptide synthesis using Fmoc-serine [PO (Obzl)OH-OH] from Calbiochem. MS-confirmed peptides are purified using RPC and a VYDAC 218TP column (purity > 98%). After having been activated with bromoacetic acid or bromoacetic-N-hydroxysuccinimide ester (Sigma), the peptides which have been prepared in this way are conjugated to thiolated KLH (keyhole limpet hemocyanin, nanoTools).

Female Balb/c mice (6 weeks old) are immunized subcutaneously at 14-day intervals with the KLH-phosphopeptide (10 µg/mouse) containing complete Freund's adjuvant in the first injection and incomplete adjuvant in the 3 following injections. The mice are then (2 weeks later) given booster injections of 10 µg of immunogen in PBS (phosphate- buffered saline) on three consecutive days. 1 day after the last booster injection, the mice are sacrificed and the spleens removed. Spleen cells are isolated and fused with non-producing myeloma cells (e.g. PAI-Zellen, J.W. Stocker et al. (1982) Res. Disclosure, 21713) using the established Köhler/Milstein methodology. A differential screening method using phosphorylated/non-phosphorylated peptide as well as phosphorylated/nonphosphorylated recombinant human VASP is employed to test hybridoma cells for their ability to secrete antibodies against phosphoserine 157 VASP.

For the test using phosphorylated/non-phosphorylated peptide, these peptides are coupled covalently to DNA-BIND-ELISA plates (from Costar) and the hybridoma supernatants are screened using the ELISA method. Supernatants which recognize phosphopeptide are additionally examined for their ability to recognize completely phosphorylated recombinant (E. coli system) human VASP but not the correspondingly dephosphorylated VASP.

Monoclonal antibodies from the supernatants of the hybridoma cells which have been identified by the above described methods as being positive can preferably be purified from serum-free hybridoma cell cultures by means of thiophilic adsorption chromatography (POROS 50-OH, nanoTools).

The isolated antibody (clone 5C6) has been characterized as a monoclonal antibody of the mouse IgG1K class which only recognizes VASP when this protein is phosphorylated at the serine 157 position. Antibody 5C6 does not recognize other proteins and other VASP phosphorylation sites under the conditions employed.

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Publication:

Smolenski et al., (2000), Regulation of Human Endothelial Cell Focal Adhesion Sites and Migration by cGMP-dependent Protein Kinase I J. Biol. Chem., 275: 25723-25732

Burkhardt et al. (2000) - KT5823 Inhibits cGMP-dependent Protein Kinase Activity *in Vitro* but Not in Intact Human Platelets and Rat Mesangial Cells J Biol Chem 275: 33536-33542

Example 2

Monoclonal antibodies which specifically recognize phosphoserine 239 VASP have been isolated in analogy with the above-described method by employing phosphorylated peptides, which encompass the known peptide sequences around serine 239 of the VASP protein (RKVS239KQE), respectively, as the antigen.

These monoclonal antibodies specifically recognizing VASP phosphorylated at position Ser 239 include antibodies VASP (pS239)-22E11B8, VASP (pS239)-22E11D3 and VASP (pS239)-22E11G10. These antibodies show similar sensitivities when compared to antibody 16C2 (Fig. 1) using sodium-nitroprusside incubated (100 μ mol/L for 2 min at 37°C) and subsequently lysed whole blood as a standard.

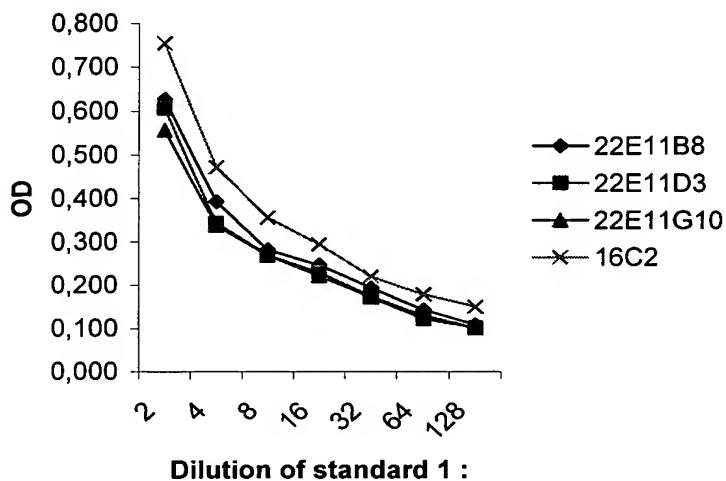


Fig. 1: Comparison of sensitivity of antibodies 22E11B8, 22E11D3 and 22E11G10 With antibody 16C2

Antibodies (pS239)-22E11B8, VASP (pS239)-22E11D3 and VASP (pS239)-22E11G10 only recognizes VASP when this protein is phosphorylated at the serine 239 position. The antibodies do not recognize other proteins and other VASP phosphorylation sites under the conditions employed.